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Phospholipid Requirement of the Vanadate-Sensitive ATPase from Maize Roots Evaluated by Two Methods

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ABSTRACT

The activation of the vanadate-sensitive ATPase from maize (*Zea mays* L.) root microsomes by phospholipids was assessed by two different methods. First, the vanadate-sensitive ATPase was partially purified and substantially delipidated by treating microsomes with 0.6% deoxycholate (DOC) at a protein concentration of 1 milligram per milliliter. Vanadate-sensitive ATP hydrolysis by the DOC-extracted microsomes was stimulated up to 100% by the addition of asolectin. Of the individual phospholipids tested, phosphatidylserine and phosphatidylglycerol stimulated activity as much as asolectin, whereas phosphatidylcholine did not. Second, phospholipid dependence of the ATPase was also assessed by reconstituting the enzyme into proteoliposomes of differing phospholipid composition. In these experiments, the rate of proton transport and ATP hydrolysis was only slightly affected by phospholipid composition. DOC-extracted microsomes reconstituted with dioleoylphosphatidylcholine had rates of proton transport similar to those found with microsomes reconstituted with asolectin. The difference between the two types of assays is discussed in terms of factors contributing to the interaction between proteins and lipids.

The vanadate-sensitive ATPase from higher plants has been the subject of intensive biochemical studies and the subject of recent review (29). The characteristics of this protein are very similar to those of other E1-E2 transport ATPases in which the reaction mechanism includes the formation of an aspartyl phosphate intermediate. The vanadate-sensitive ATPase from higher plants is more similar to the E1-E2 of ATPase from fungi because both catalyze electrogenic proton transport (7). Interest in this protein originates mostly from its suspected role in ion transport. According to chemiosmotic concept (7, 11, 29), the ATPase converts energy from ATP hydrolysis to generate a proton motive force which drives transport of other solutes.

In recent years, interest in the vanadate-sensitive ATPase has expanded to include its role in physiological events such as adaptation to environmental conditions, and growth and development. There are at least four possible ways that activity of the ATPase can be regulated: (a) gene regulation (30); (b) protein modification including phosphorylation (2); (c) allosteric like regulation by either plant hormones or other small molecules (17, 25); and (d) alterations in the lipid environment surrounding the ATPase. Adaptation of plants to cold and the development of senescence have been associated with changes in fatty acid composition of membrane lipids and to a lesser extent types of polar lipids (3, 18) as well as changes in ATPase activity and maintenance of solute gradients (9, 12, 28). These results possibly indicate that modulation of the lipid environment regulated ATPase activity. Plant responses to pathogens can involve either the activation or inhibition of the enzyme with fusicoccin probably being the most extensively studied phytotoxin effecting the ATPase activity (20). Modulation of the ATPase by this toxin appears to be complex and indirect since the reputed fusicoccin binding protein was purified away from plasma membrane ATPase activity (27). Cytokinins, auxins, and ABA alter membrane fluidity and permeability (8, 13, 26), which may indirectly effect ATPase activity. It is clear that modulation of vanadate-sensitive ATPase activity probably proceeds via numerous responses to environmental and developmental changes. However, changes in the membrane lipid composition clearly remains as one of the key mechanisms likely to be involved in the regulation of ATPase activity.

Vanadate-sensitive ATPases purified from plants and other organisms appear to have specific lipids requirements (11, 15). In general, the lipid dependence of partially purified or purified membrane-bound enzyme can readily be studied because the detergents used during purification of the enzyme usually remove most of the endogenous lipids and the activity of the partially purified or purified enzyme is either stimulated or dependent on the addition of exogenous phospholipids (11). Partially purified ATPase from higher plants is no exception to the above generalities (10, 15). In most research to date, the lipid dependence of ATPases has been evaluated by the effect on ATP hydrolysis but not its coupled transport activity. Yet, the lipid requirements for ATP hydrolysis and ion transport may indeed be different. For example, the proton pump from clathrin-coated vesicles required PS[1] for maximal rates of ATP hydrolysis, but not for proton transport by the reconstituted ATPase (32). In this report, the effect of phospholipid head groups on ATP hydrolysis catalyzed by the vanadate-sensitive ATPase partially purified from corn root microsomes was compared to the phospholipid require-

[1] Abbreviations: PS, phosphatidylserine; AO, acridine orange; BTP, Bis-Tris-Propane; DGDG, digalactose diacylglycerol; DOC, sodium salt of deoxycholate; DOPC, dioleoylphosphatidylcholine; DPPC, dipalamitoylphosphatidylcholine; k_l, proton leakage from vesicles while the proton pump is catalyzing transport; MGDG, monogalactose diacylgalactose; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

ment of ATP hydrolysis and its coupled transport process by reconstituted enzyme.

MATERIALS AND METHODS

Isolation of Membrane Preparations

KI-washed microsomes were isolated from 3-d-old maize (*Zea mays* L.) roots grown on filter paper moistened with 0.1 mM $CaCl_2$ as described previously (4). The vanadate-sensitive ATPase was partially purified from the microsomes by treatment with DOC. Microsomes were diluted with resuspension buffer (3 mM BTP-Mes [pH 7.2], 10% [w/v] glycerol, 0.25 M sucrose, 2 mM DTT) to a protein concentration of 1 mg/mL. To this solution, 10% (w/v) DOC was added to a final concentration of 0.6%. After 10 min on ice, the solution was centrifuged at 38,000 rpm in a Ti 70 rotor for 75 min. The resulting pellet was dispersed in resuspension buffer. The centrifugation step was repeated and this final pellet was dispersed again in resuspension buffer at approximately 1 mg protein/mL. The vanadate-sensitive ATP hydrolysis in the DOC-extracted microsomes could be stored at least two weeks at −20°C without a loss of enzymatic activity. Protein concentration was determined after precipitation by TCA in the presence of DOC by the Lowry method (1).

The vanadate-sensitive ATPase in KI-washed microsomes and DOC-extracted membranes was reconstituted by the protocol described previously (4). Briefly, 0.1 to 1 mg of membrane protein were combined with 1 to 12 mg of phospholipids in a total volume of 1 mL, dispersed by the addition of 70 μL of 10% (w/v) DOC and applied to a Sephadex G-150 column (1 × 19 cm). Proteoliposomes emerged in the void volume and were stored on ice until assayed. Phospholipids were partially purified from asolectin and stored as described by Kagawa and Racker (14). Analysis of asolectin by TLC indicated that PC comprised 50% of the lipids present. Substantial quantities of PI, PE, PS, and lyso-PC were also found as well as trace amounts of other polar lipids (data not shown). Synthetic DOPC and DPPC of 99% purity, PA, PE, and PG of greater than 98% purity (from egg yolk), PS of 98% purity (from bovine brain) and PI of 99% purity (from soybean) were obtained from Sigma Chemical Co.

Assays of ATPase Activities

Proton transport was followed by changes in the absorbance of AO at 492 nm as described previously (4). Typically, 200 μL of vesicles were diluted with 2 mL of 17.5 mM Mes-BTP (pH 6.45), 2.5 mM $MgSO_4$, 1 mM EGTA, 7.5 μM AO and 50 mM KNO_3. After equilibration at the temperature of the assay (18–22°C) for 5 min, the reaction was initiated by the addition of 20 μL of 0.2 M ATP titrated to pH 6.45 with BTP. Where indicated, the kinetics of proton transport were analyzed according to the method of Tu *et al.* (31). Recently, proton transport by the vanadate-sensitive ATPase from maize root microsomes was shown to conform to this model (5). This model allows one to quantify the overall proton transport process by simultaneously considering the pumping rate and the leakage of protons from membrane vesicles. Of importance, the rate of proton leakage in the direction opposite from the pump while the pump is catalyzing proton transport, k_1, is estimated from the relationship $\ln(1-\Delta A_t/\Delta A_s) = k_1 \cdot t$, where ΔA_t is the net change in AO absorbance at time t, ΔA_s is the steady-state change in AO absorbance and t is time. The initial rate of proton transport, R_0, is found by $R_0 = k_1 \cdot \Delta A_s$.

ATP hydrolysis was assayed using 5 to 10 μL of either KI-washed microsomes or DOC-extracted microsomes diluted to 100 μL with proton transport reaction media lacking AO but containing 0.02% Triton X-100. Activity was assessed either by the amount of inorganic phosphate release as determined by the formation of the malachite green-molybdate complex (31) or changes in the absorbance at 340 nm following the oxidation of NADH in the presence of pyruvate kinase, lactate dehydrogenase and PEP as described previously (31). Phospholipid vesicles prepared by exhaustive sonication (3 × 5 min) under N_2 at a concentration of 2 mg/mL were added to the assay medium as indicated in the text. Vanadate-sensitive ATPase activity was determined by measuring the difference between the rate of ATP hydrolysis in the absence and presence of 0.2 mM sodium orthovanadate.

Lipid Analysis

Polar lipids were extracted from aqueous suspensions and purified by TLC by the protocol of Moreau and Isett (21). Spots which chromatographed like corresponding phospholipid standards were scraped from the plate and subjected to total phosphate analysis by the method of Dittmer and Wells (6). Spots which chromatographed like corresponding galactolipids were also scraped from the plate and subjected to reducing sugar analysis by the method of Roughan and Batt (23) using galactose as a standard. Total phospholipid content was determined after organic solvent extraction by subjecting the dried lipid film to total phosphate analysis without purification by TLC.

Neutral lipids were separated by TLC using Whatmann[2] LHP-K plates developed sequentially in 80:20:1.5 (v:v:v) hexane:ether:acetic acid followed by 80:20 hexane:ether. Spots were visualized by dipping in the plate in 10% (v/v) H_3PO_4 and 10% (w/v) $CuSO_4 \cdot H_2O$ followed by 15 min incubation at 120°C. Quantitation was performed by densitometry using CAMAG TLC Scanner II.

RESULTS AND DISCUSSION

Characterization of DOC-Extracted Microsomes

After treating KI-washed microsomes with 0.6% DOC, less than 30% of the total protein (1.0 mg out of an initial 3.6 mg) was recovered in the 100,000*g* pellet. When assayed under standard conditions, the total vanadate-sensitive ATPase activity in the pellet and supernatant was 116 and 41 nmol/min, respectively, out of an initial total activity of 298 nmol/min. Thus, only half of the ATPase activity was recovered in the two components, indicating that considerable inactivation of the enzyme had occurred. The total phospholipid content of the microsomes was reduced by over 90% from approxi-

[2] Reference to brand and firm does not constitute endorsement by U.S. Department of Agriculture over others of similar nature.

mately 1000 nmol of phospholipid Pi to less than 50 nmol. In addition, the galactolipids, MGDG and DGDG, were likewise reduced from 77 and 256 nmol, respectively, to less than 3 and 11 nmol, respectively, after the DOC treatment. The DOC treatment reduced the sterol content to less than 1% of the level found in KI-washed microsomes (data not shown). Therefore, this treatment removed a majority of the lipid constituents. It was suspected that this loss of lipids may have resulted in an inactivation of the ATPase. When delipidated membrane fractions were assayed in the presence of 200 μg/mL asolectin, the ATPase activity was enhanced by approximately 100% from 116 nmol/min of activity in the absence of asolectin, and 221 nmol/min in its presence. The ATPase activity of KI-washed microsomes (not detergent treated) and the DOC supernatant was not affected by the addition of asolectin (data not shown). These results indicated that the vanadate-sensitive ATPase in the DOC-extracted microsomes required added lipid components present in asolectin for optimal rates of ATP hydrolysis. When the ATPase activity in the presence of asolectin was compared with that by KI-washed microsomes, almost 90% of the initial activity could be accounted for in the DOC-extracted microsomes and the DOC solubilized material. The high recovery of activity in the two fractions indicated that most of the ATPase was reactivated. Over 75% of the activity in the KI-washed microsomes was recovered in the DOC-extracted microsomes. The specific activity for the vanadate-sensitive ATP hydrolysis when assayed under optimal conditions (*i.e.* in the presence of asolectin) was enhanced 2.6- to 2.9-fold by the DOC treatment.

As prepared, the vanadate-sensitive ATPase in DOC-extracted microsomes did not catalyze proton transport. One potential reason for this lack of activity was that the DOC-extracted microsomes were not vesicular. Such an explanation seemed plausible because of the low amount of lipid present. In fact, plasma membranes extracted with 0.6% from *Neurospora crassa* can assume an open-sheet structure (7). Triton stimulation and proteolytic inactivation of a membrane-bound enzyme may indicate the presence of activity localized on the internal leaflet of the bilayer (16, 24). Results solely based on Triton X-100 stimulation should be interpreted with caution in light of more recent experiments, which suggested that Triton stimulation may reflect activation rather than orientation (24). ATP hydrolysis by KI-washed microsomes was stimulated 60 to 70% by the addition of 0.02% (w/v) Triton X-100, whereas there was no stimulation of ATP hydrolysis with DOC-extracted microsomes (data not shown). The presence or absence of asolectin had no effect on the degree of Triton stimulation of ATP hydrolysis (data not shown). ATP hydrolysis by DOC-extracted microsomes was eliminated after incubating with 0.2 mg/mL of trypsin for 10 min (data not shown). With KI-washed microsomes at a similar level of ATPase activity, all of the activity present when assayed in the absence of Triton X-100 was eliminated after 10 min of trypsin digestion but none of the Triton stimulated activity was lost. Further decreases were not evident even when the digestion time was increased to 30 min (data not shown). Thus, the results from Triton X-100 stimulation along with that from proteolytic inactivation suggested that the accessibility of the ATPase was different between KI-washed and DOC-extracted microsomes, and that the DOC-extracted microsomes were not vesicular.

Effect of Different Phospholipids on ATP Hydrolysis by the DOC-Extracted Microsomes

The ability of a variety of phospholipids to increase the activity of the vanadate-sensitive ATPase was explored (Table I). In this experiment, the addition of asolectin stimulated ATP hydrolysis 71% from 168 to 288 nmol/min/mg protein. The addition of PA enhanced activity by 95%, while PG and PS yielded levels that were similar to asolectin. The two types of PC, PI, and PE restored little or no activity.

There have been previous reports on the ability of different phospholipids to activate partially purified, vanadate-sensitive ATPase from other plant tissues (10, 15). In the case of the ATPase from rose cells partially purified by washing plasma membranes with cholate, very little activity was observed in the absence of polar lipids (10). The addition of PI, PC, and PE did not enhance activity whereas PG and PS greatly stimulated ATPase hydrolysis. Kasamo and Nouchi (15) reported that the highly purified ATPase from mung beans was activated by PS. However, with the mung bean enzyme, PC stimulated activity almost to the same extent as PS. PI and PG supported intermediate levels of activity, and PA and PE were not stimulatory. The proton-translocating ATPases from fungi show a dependence on either acidic phospholipids or PC depending on the organism from which the ATPase was purified (7). Acidic phospholipids may be required to activate enzymes that transport protons (7).

Further experiments compared the effects of asolectin, DOPC and PG on activity because these lipids represented the extremes in their ability to activate the ATPase. Activation was concentration dependent (Fig. 1). When either asolectin or PG were tested at a protein concentration of 38 μg/mL, these phospholipids showed little effect below 2 μg/mL and a dramatic enhancement of activity at lipid concentrations above 100 μg/mL. Increasing concentrations of DOPC resulted in slight stimulation of ATP hydrolysis throughout the concentration range tested.

Table I. *Effect of Different Phospholipids Added at 200 μg/mL on the Vanadate-Sensitive ATPase Activity of DOC-Extracted Microsomes*

ATPase activity was assessed in the absence of Triton X-100 as described in "Materials and Methods." The activity relative to no lipid added is presented within the parentheses.

Phospholipid Added	Vanadate-Sensitive ATPase Activity
	nmol Pi/min·mg protein
None	168
Asolectin	288 (1.71)
PA	328 (1.95)
PG	296 (1.76)
PS	273 (1.63)
PI	206 (1.23)
PE	181 (1.08)
DOPC	183 (1.09)
DPPC	164 (0.98)

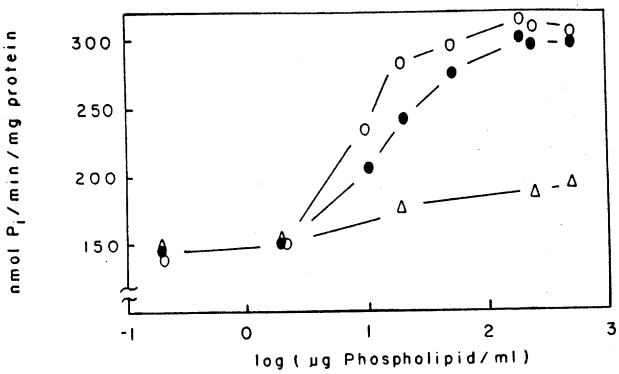

Figure 1. Effect of the concentration of asolectin, DOPC, or PG on vanadate-sensitive ATP hydrolysis by DOC-extracted microsomes. Vanadate-sensitive ATPase activity was assessed in the absence of Triton X-100, and in the presence of varying concentrations of asolectin (○), DOPC (△) and PG (●) at a protein concentration of 38 µg/mL. When assayed in the absence of added phospholipid, the vanadate-sensitive ATPase activity was 141 nmol/min/mg protein.

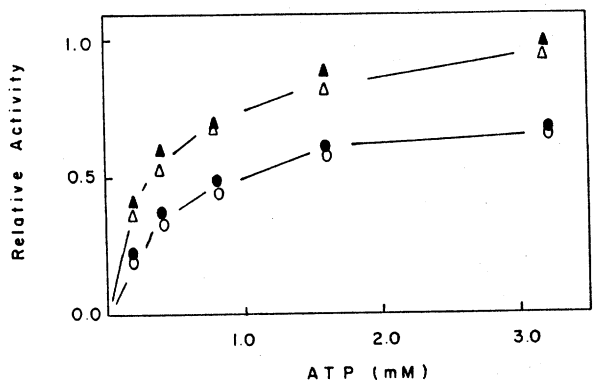

Figure 2. ATP dependence of vanadate-sensitive ATP hydrolysis by DOC-extracted microsomes in the absence and presence of added phospholipids. Vanadate-sensitive ATPase was assayed in the absence of added lipid (○), and in the presence of 200 µg/mL of asolectin (▲), DOPC (●) or PG (△), using ATP concentrations from 0.2 to 3.2 mM in the presence of 4 mM $MgSO_4$. The activity is plotted relative to that found in the presence of asolectin at 3.2 mM ATP, which was 285 nmol Pi/min/mg protein.

The kinetics of ATP hydrolysis by DOC-extracted microsomes in the absence of added lipids was compared to that in the presence of 200 µg/mL of asolectin, DOPC or PG. In each case, the kinetics with respect to ATP conformed to a simple Michealis-Menten relationship (Fig. 2). The K_m values were relatively constant ranging from 0.39 mM in the presence of asolectin to 0.48 mM in the presence of DOPC with intermediate values of 0.42 and 0.44 mM in the presence of PG and in the absence of added lipids, respectively. These K_m values for ATP were similar to the ones reported previously by our laboratory for KI-washed microsomes (4). The V_{max} was approximately the same in the absence of lipid and in the presence of DOPC averaging 130 nmol/min/mg protein. The addition of PG and asolectin increased the V_{max} to 289 and 325 nmol/min/mg protein, respectively. These results indicated that lipids stimulated the rate of turnover of the enzyme but did not have a significant effect on K_m for ATP.

The following experiment was conducted to determine if activation of the vanadate-sensitive ATPase in the DOC-extracted microsomes was due to the formation of a mixed micelle with the added phospholipids. Two mL of reaction mixtures with or without DOC-extracted microsomes in the presence and absence of either DOPC or PG were incubated for 10 min at room temperature. After cooling on ice to 4°C, the reaction mixture was layered over a 5 to 30% (w/v) glycerol gradient in 17.5 mM Mes-Tris (pH 6.45) containing 1 mM EGTA. After centrifuging for 16 h at 25,000 rpm in SW 40 rotor, the gradients were fractionated into 0.8 mL aliquots which were analyzed for phospholipid content and vanadate-sensitive ATPase activity. Without DOC-extracted microsomes present, well-sonicated vesicles prepared from either DOPC or PG accumulated near to top of the glycerol gradient. In the absence of added phospholipids, DOC-extracted microsomes pelleted through the gradient since virtually all of the protein and ATPase activity was recovered in the pellet (data not shown). All of the vanadate-sensitive ATPase activity was recovered in the pellet in the presence of either DOPC or PG vesicles. These results suggested that the activation of the vanadate-sensitive ATPase in DOC-extracted microsomes by phospholipids did not involve the formation of a mixed micelle between the lipids and the proteins. Activation of the H^+-ATPase from *N. crassa* plasma membrane also did not involve the protein binding to lipid micelles (7). The results here and from *N. crassa* differ from that of the H^+-ATPase of yeast plasma membrane where activation of the purified protein by PC occurred concomitantly with binding to lipid micelles (7).

During the course of these experiments it was observed that there was an inverse correlation between the ability of different phospholipids to stimulate ATP hydrolysis and their ability to scatter light, *i.e.* those phospholipids that scattered more light, did not activate the ATPase (data not shown). Differences in light scattering at the same concentration of phospholipids is related to differences in the size of the particles in solution (25). Thus, the association between light scattering and ability to activate the ATPase may indicate that the size of the vesicles of the different classes of phospholipids determine the degree of activation of the ATPase. To reduce the likelihood that the inability of particular phospholipids, such as PC, to activate the ATPase resulted from either the size of the vesicles or other physical characteristics of the lipid aggregate, the phospholipid dependence of reconstituted ATPase was examined. Because the ATPase would be incorporated into a lipid bilayer, there was less of a chance that the physical properties of the phospholipid vesicles would be a determinant of activation.

Phospholipid Dependence of the Reconstituted ATPase

The vanadate-sensitive ATPase in both KI-washed and DOC-extracted microsomes could be reconstituted into proteoliposomes using the protocol described previously (4). The recovery of both ATP hydrolysis and proton transport activities after reconstitution was dependent on the amount of lipid added (Fig. 3). In the case of KI-washed microsomes reconstituted with asolectin, the rates of ATP hydrolysis and proton transport both increased similarly as a sigmoidal func-

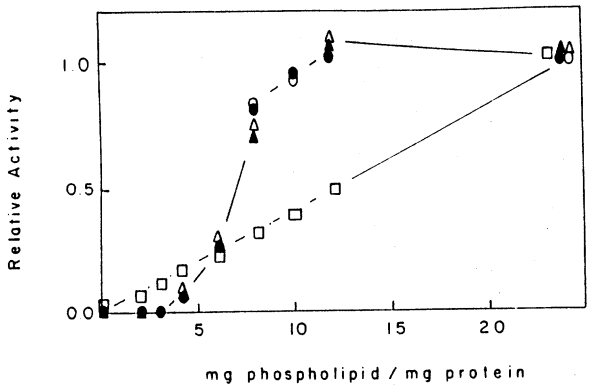

Figure 3. Effect of the lipid to protein ratio on the recovery of phospholipid and ATPase activities of reconstituted KI-washed microsomes. KI-washed microsomes were reconstituted with varying amounts of phospholipid as asolectin, DOPC or a 5:1 (w/w) mixture of DOPC to PG. Proteoliposomes formed from asolectin were assayed for total phospholipid (□), vanadate-sensitive ATP hydrolysis (△) and nitrate-insensitive proton transport (▲). All results were plotted as a function of the value found with proteoliposomes formed from asolectin with a phospholipid to protein ratio of 24. The values for total phospholipid, vanadate-sensitive ATP hydrolysis and proton transport of the vesicles reconstituted with asolectin at a lipid to protein ratio of 24 to 1 were 20.2 mg, 102 nmol Pi/min·mg protein and 0.105 A/min·mg protein, respectively. KI-washed vesicles reconstituted with either DOPC (○) or a 5:1 (w/w) mixture of DOPC:PG (●) were assayed for nitrate-insensitive proton transport.

tion of the added lipid saturating above a ratio of 10:1 (w/w) lipid to protein. The amount of phospholipid recovered after reconstitution was linearly related to the amount added with a slope of about 0.75.

The recovery of proton transport activity was similar when the asolectin was replaced with DOPC (Fig. 3). Results analogous to those found with asolectin were observed for the recovery of phospholipids and ATP hydrolysis when KI-washed microsomes were reconstituted with DOPC (data not shown). It was difficult to reconstitute the ATPase in DPPC liposomes because this phospholipid (which contains only saturated fatty acids) did not readily form liposomes by our method. When 1 mg of protein from KI-washed microsomes was reconstituted with 12 mg of DPPC, less than 3 mg of phospholipids were recovered and no enzyme activity was observed. A recovery of 3 mg of phospholipid was not sufficient to recover ATPase activities (Fig. 3). The inability to form liposomes with DPPC may be related to differences in the lipid phases, since at room temperature DPPC is below its transition temperature and presumably in a gel phase, whereas DOPC is above its transition temperature and presumably in a liquid crystalline phase (11). Because of the difficulties in forming vesicles made of certain phospholipids, like DPPC, the ability of different phospholipid headgroups to activate the ATPase was tested in mixtures with DOPC. Using the stimulation of ATP hydrolysis by DOC-extracted microsomes as a guide, it was found that a ratio of 1 part PG to 5 parts DOPC was sufficient to fully stimulate ATPase activity (Table II). When KI-washed microsomes were reconstituted in a 5:1 mixture of DOPC and PG, the recovery of

Table II. *Effect of Selected Phospholipids on the ATPase Activities of DOC-Extracted Microsomes and Reconstituted DOC-Extracted Microsomes*

Phospholipids were added to a final concentration of 400 μg/mL when assaying ATP hydrolysis by DOC-extracted microsomes. DOC-extracted microsomes were reconstituted using 36 mg of lipid per mg of protein using asolectin, DOPC or a mixture of 5:1 (w/w) DOPC to PG.

Phospholipid Added	Vanadate-Sensitive ATP Hydrolysis		Proton Transport by Reconstituted Vesicles
	DOC vesicles	Reconstituted	
	nmol Pi/min·mg protein		*A/min·mg protein*
No lipid added	135	0	0
Asolectin	290	264	0.285
DOPC	165	245	0.302
PG	281	nd	nd
5 DOPC + 1 PG	274	274	0.339

proton transport activity was similar to that found with asolectin and DOPC.

With KI-washed microsomes reconstituted into proteoliposomes, DOPC activated proton transport by the ATPase to the same extent as asolectin (Fig. 3), whereas asolectin activated the ATPase to a greater extent than DOPC when phospholipids were added to the DOC-extracted microsomes (Fig. 1). This dissimilarity in the activation of the ATPase by DOPC may suggest that factors other than phospholipid head influenced the ability of certain classes of phospholipids to activate the ATPase depleted of endogenous phospholipids. Such a conclusion is dependent on a low recovery of endogenous lipids when KI-washed microsomes were reconstituted with DOPC. To assess the recovery of endogenous lipids during reconstitution, 1 mg of protein from KI-washed microsomes was reconstituted with 12 mg of DOPC, and the resulting proteoliposomes were analyzed for galactolipids. The galactolipids found in reconstituted vesicles originated only from the KI-washed microsomes since the microsomes contained both MGDG and DGDG while the DOPC did not. The KI-washed microsomes contained 22 and 73 nmol galactose/mg protein as MGDG and DGDG, respectively, prior to reconstitution and the resulting reconstituted vesicles contained less than 1 and 4 nmol galactose/mg protein as MGDG and DGDG, respectively. These data suggest that less than 5% of the endogenous lipids were recovered after reconstitution at saturating levels of added phospholipid. The recovery of PE and sterols in reconstituted DOPC vesicles were similar to that found for MGDG and DGDG (data not shown). Therefore, activation of ATPase in reconstituted vesicles was probably caused by exogenous DOPC and not due to the presence of endogenous lipids.

The effect of phospholipids on the recovery of ATPase activity from DOC-extracted microsomes should be a better system for evaluating the phospholipid dependence since this enzyme preparation was significantly depleted of lipids prior to reconstitution. The ATPase in DOC-extracted microsomes could be incorporated efficiently into phospholipid vesicles by the reconstitution protocol. In the case of KI-washed

microsomes, under optimum conditions essentially all of the vanadate-sensitive ATP hydrolyzing activity could be recovered after reconstitution. When 0.24 mg of DOC-extracted microsomes containing 70 nmol Pi released/min of vanadate-sensitive ATP hydrolysis (assayed in the presence of 200 μg/mL asolectin) were reconstituted with 12 mg of asolectin, the resulting proteoliposomes had 68 nmol Pi/min of total activity. The recovery of proton transport activity could not be assessed directly since the ATPase did not exhibit proton transport prior to reconstitution but was inferred from the increase in the specific activity for proton transport by reconstituted DOC-extracted microsomes as compared to that of KI-washed microsomes. For example, the specific activity for proton transport by reconstituted vesicles increased 2.6-fold from 0.106 A/min·mg protein with KI-washed microsomes to 0.282 with DOC-extracted microsomes. With these preparations, the vanadate-sensitive ATPase activity increased 2.7-fold from 108 nmol Pi/min·mg protein to 292 nmol Pi/min·mg protein when assayed in the presence of 200 μg/ml asolectin. Thus, the increase in specific activity in reconstituted proton transport was similar to the increase in the specific activity of vanadate-sensitive ATP hydrolysis. These results argue in favor of a high recovery of proton transport activity when the DOC-extracted vesicles were reconstituted.

In comparison to KI-washed microsomes, a higher lipid to protein ratio was required for optimal rates of proton transport when reconstituting DOC-extracted microsomes (Fig. 4). Very little proton transport was recovered at phospholipid to protein ratios below 10 to 1 and recovery saturated at ratios above 40 to 1. When the recovery of proton transport activity was plotted as a function of the ratio of phospholipid to ATPase activity, results from KI-washed and DOC-extracted microsomes were identical (Fig. 5). These results suggested that the recovery of ATPase activity was determined by the ratio of phospholipids to ATPase, independent of the membrane preparation.

When asolectin was replaced with either DOPC alone or a 5:1 (w/w) mixture of DOPC:PG, the resulting proteoliposomes had similar rates of proton transport. Therefore, the ATPase reconstituted from the DOC-extracted microsomes

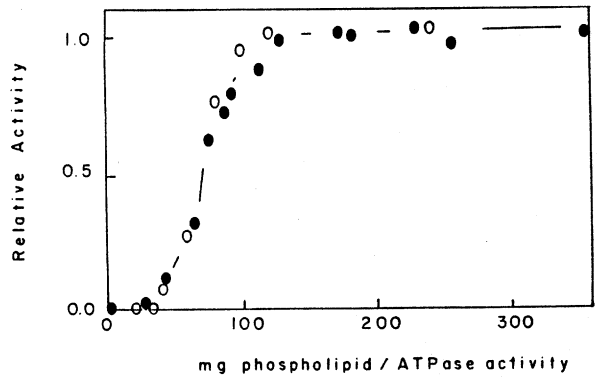

Figure 4. Effect of the lipid to protein ratio on the proton transport activity for reconstituting the ATPase in DOC-extracted microsomes. DOC-extracted microsomes were reconstituted and then assayed for nitrate-insensitive proton transport using varying amounts of asolectin (△), DOPC (●) or the 5:1 (w/w) mixture of DOPC:PG (○).

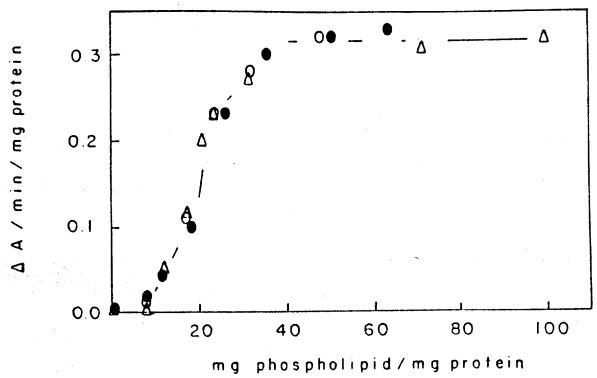

Figure 5. Lipid dependence for the reconstitution of KI-washed and DOC-extracted microsomes expressed in terms of ATPase activity. The data from Figures 3 and 4 for KI-washed (○) and DOC-extracted microsomes (●), respectively, were averaged across the three phospholipid sources and the activity relative to that found with ATPase reconstituted at the highest asolectin ratio was plotted as a function of the ratio of phospholipid added for reconstitution to the ATPase activity as μmol of Pi/min·mg protein of vanadate-sensitive ATP hydrolysis. The specific activity for KI-washed and DOC-extracted microsomes corresponding to a relative activity of 1.0 was 0.105 and 0.32 A/min/mg protein, respectively.

Table III. *Effects of Various Phospholipid Combinations on the Activity of the Vanadate-Sensitive ATPase in DOC-Extracted Microsomes after Reconstitution*

DOC-extracted microsomes were reconstituted with asolectin, DOPC or DOPC in combination with other phospholipids at a ratio of 5:1 (w/w) using 40 times as much lipid as protein on a weight basis. The resulting proteoliposomes were assayed for ATP hydrolysis and proton transport. Proton transport data were analyzed by a kinetic model as described previously (30) to estimate k_1, the rate of proton leakage from the vesicles during pumping.

Lipid Added	Vanadate-Sensitive ATP Hydrolysis	Kinetic Components of Proton Transport		
		R_0 initial rate of proton uptake	k_1 proton leakage	ΔA_e extent of proton uptake
	nmol/min·mg protein	*ΔA/min·mg protein*	*min⁻¹*	*ΔA/mg protein*
Asolectin	304	0.30	0.29	1.06
DOPC	295	0.30	0.29	1.07
DOPC + PI	298	0.32	0.26	1.21
DOPC + PE	310	0.33	0.26	1.26
DOPC + PG	308	0.33	0.26	1.27
DOPC + PA	304	0.34	0.27	1.25
DOPC + PS	301	0.38	0.20	1.89

was activated by DOPC as other phospholipids in a fashion similar to that found for the reconstitution of KI-washed microsomes.

A direct comparison between the activation of vanadate-sensitive ATP hydrolysis in DOC-extracted microsomes by asolectin, DOPC and DOPC-PG and the requirement of DOC-extracted microsomes for these lipids during reconstitution was made (Table II). The addition of 400 μg/mL asolectin to the ATP hydrolysis assay media increased the activity from 135 to 290 nmol Pi/min·mg protein. Similar rates of ATP hydrolysis were found when the asolectin

was replaced with PG. The addition of DOPC stimulated ATP hydrolysis only slightly. The inclusion of 5:1 (w/w) DOPC:PG at a final lipid concentration of 400 μg/ml was sufficient to stimulate ATPase activity almost to the level with PG alone. When the DOC-extracted microsomes were reconstituted at a protein to lipid ratio of 40 to 1, the resulting proteoliposomes had similar rates of both ATP hydrolysis and proton transport. The resulting proteoliposomes were assayed for their phospholipid content and the recovery of individual lipids were comparable to that found for asolectin in Figure 3 (data not shown). These results also indicated that the vanadate-sensitive ATPase did not have the same lipid requirement when assayed for the stimulation of ATP hydrolysis and reconstitution.

Reconstitution further reduced the presence of native lipids in the DOC-extracted microsomes. Prior to reconstitution in DOPC at a lipid to protein ratio of 40, the DOC-extracted vesicles had 12 and 1 nmol of galactose per mg protein as DGDG and MGDG, respectively, and less than 1 nmol of galactose/mg protein of each lipid after reconstitution. This low recovery suggests that a substantial amount of the endogenous lipids did not remain after the ATPase was partially purified by DOC treatment and then reconstituted.

A range of different lipids were tested for their ability to reconstitute the ATPase in DOC-extracted microsomes when incorporated with DOPC (Table III). Different phospholipids were combined with DOPC to yield a ratio of 5 parts of DOPC and 1 part of tested lipid at a protein to lipid ratio of 40 to 1 and compared to proteoliposomes made with asolectin. The kinetics of proton transport were analyzed as described previously (5, 31), so that the rate of proton leakage from vesicles during pumping could be estimated. Rates of vanadate-sensitive ATP hydrolysis by reconstituted DOC-extracted microsomes were independent of the phospholipid used during reconstitution. The initial rate of proton transport varied slightly from 0.30 to 0.34 A/min·mg protein except for the highest rate of 0.38 A/min·mg protein with the combination of DOPC and PS. The high rate of proton transport in the presence of PS may not have reflected activation of the ATPase by PS but rather a reduced rate of proton leakage during transport. Among the phospholipids tested, proton transport rates were greatest when the rate of proton leakage was least. Except possibly for proton transport in the presence of PS, there was little evidence to suggest that the reconstituted ATPase had a requirement for specific phospholipids. The resulting proteoliposomes were analyzed for phospholipid content, and recoveries of phospholipids among the different combinations were similar to that found for asolectin in Figure 3 (data not shown). The addition of Triton X-100 stimulated ATPase activity to the same extent in each of the different proteoliposome preparations (data not shown). If one considers Triton X-100 stimulation as a measure of inside-out vesicles (16), the lack of differences in Triton X-100 stimulation of ATPase activity among the different proteoliposomes argues in favor of the same proportion of inside-out vesicles among the different reconstituted vesicles. Although partially purified ATPase from other plant sources have been reconstituted (27), the phospholipid used in these previous studies was only asolectin and therefore the phospholipid dependence was not assessed.

CONCLUSIONS

In this study, the phospholipid dependence of the vanadate-sensitive ATPase was assessed by two different means. Using the first method, the ability of phospholipids to stimulate ATP hydrolysis in a partially purified preparation depleted of lipids by detergent extraction was examined. The results of these investigations suggested that certain phospholipids were better activators of ATP hydrolysis. In particular, the results suggested that the ATPase was not activated by PC. Using the second method, when phospholipid dependence was assessed by reconstituting the ATPase, the results indicated that there were only slight differences in the activity of ATP hydrolysis and proton transport between proteoliposomes made of different phospholipids. Thus, results from the reconstitution assays indicated that PC could activate the ATPase. With regard to lipid activation, the vanadate-sensitive proton pump from maize roots appeared to be more similar to the proton pump from yeast than *N. crassa* (7). These results are consistent with a recent finding in our laboratory that lyso-PC can activate a more highly purified ATPase (A-F Hsu, unpublished data). The differences in the conclusions between the two types of experiments may indicate that some other factors besides phospholipid headgroup influenced the ability of a phospholipid to activate a membrane-bound enzyme when added to partially purified preparations that have been depleted of endogenous lipids by detergent treatments. The aggregate, *i.e.* three dimensional shape, formed by phospholipids is in part dependent on the phospholipids used (11). Also, phospholipids differ in their critical micelle concentration (11). Both of these factors may have influenced the ability of a particular phospholipid to activate the ATPase when added to the assay medium, especially in cases where activation does not involve binding of the enzyme to a lipid micelle. One ideal situation would be to study phospholipid activation at a lipid concentration below the critical micelle concentration where the interaction would be between the enzyme and lipid monomers. In the case of reconstitution, the ATPase has been incorporated into a lipid bilayer, which should minimize complications arising from the physical properties of the lipid aggregate. However, differences in the ability of different phospholipids to form liposomes by different reconstitution methods may place constraints on the types of experiments possible. The results of this study clearly indicate that data concerning phospholipid activation of membrane-bound enzymes should be interpreted in light of the methods used to assess the lipid-protein interaction and the characteristics of the lipids employed.

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